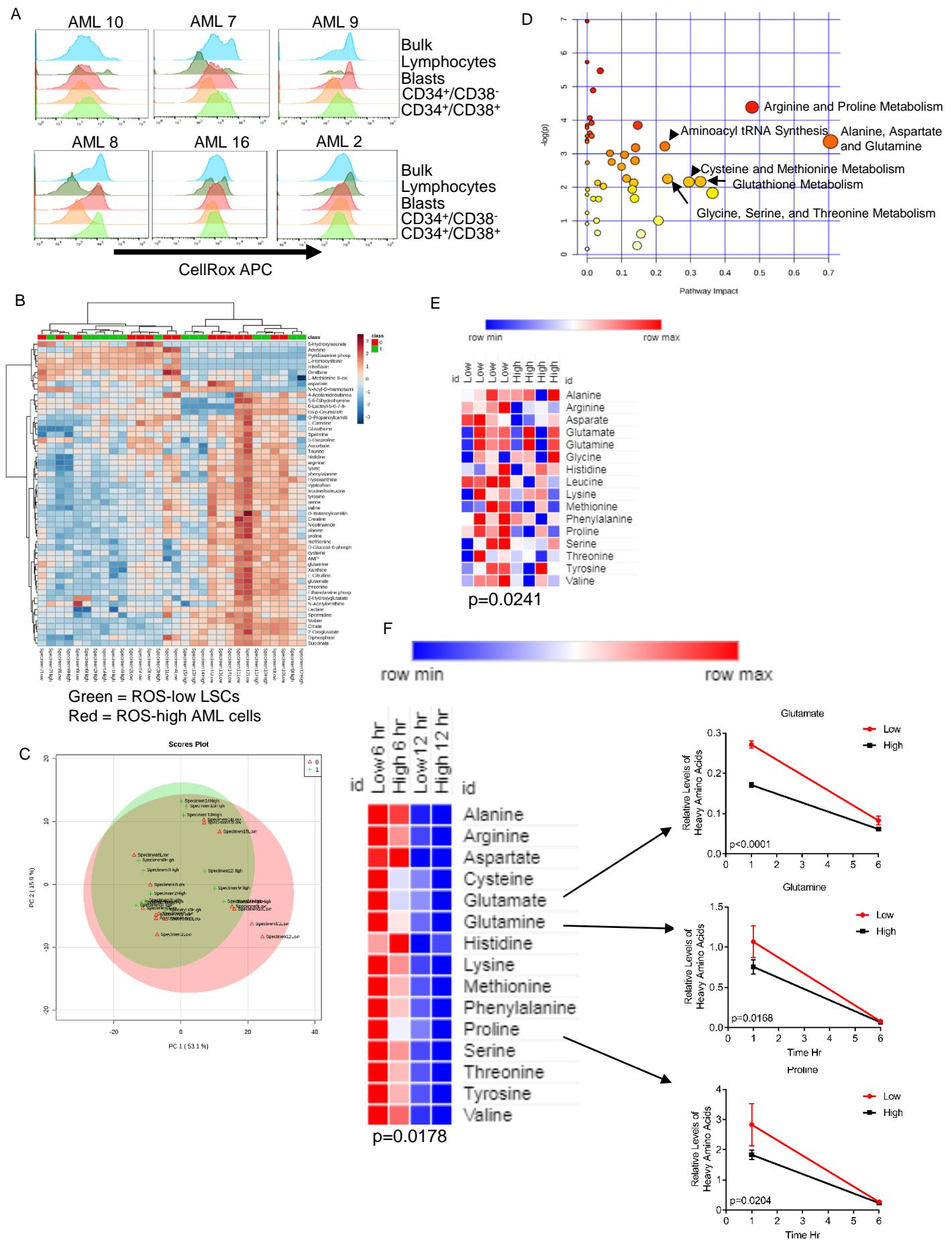


Supplemental Information

**Inhibition of Amino Acid Metabolism Selectively Targets Human Leukemia Stem Cells**

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**Figure S1: Metabolic Changes in ROS-low LSCs and ROS-high cells. Related to Figure 1.**

A. ROS levels in CD34<sup>+</sup>/CD38<sup>-</sup>, CD34<sup>+</sup>/CD38<sup>+</sup>, total blasts, lymphocytes, and bulk product. B. Top 50 individual metabolites in ROS-low LSCs (Green) and ROS-high cells (Red) ranked by p-value. **Statistical significance was determined by paired two-tailed Student's t-test.** C. PCA analysis of ROS-Low LSCs and ROS-high blasts. D. Pathway analysis of metabolites with differential abundance in ROS-low LSCs and ROS-high AML blasts determined using Metaboanalyst 4.0. E. Heatmap of amino acid uptake after 1 hr culture with stable isotope labeled amino acids, using specimen 8. **Statistical significance was determined by an unpaired two-tailed Student's t-test.** F. Heatmap of amino acid metabolism in ROS-low LSCs and ROS-high blasts isolated from patients 2 after a 6 hr or 12 hr washout of stable isotope-labeled amino acids with examples of glutamine, glutamate, and proline metabolism. Linear regression analysis was performed to identify **statistically significant** differences in amino acid metabolism between ROS-low LSCs and ROS-high blasts.

**Table S1: Patient Characteristics. Related to Figure 1.**

Patient Sample	Diagnosis	Age/Sex	Cytogenetics	Mutations
1 Refractory		47/M	46,XY,del(7)(q21)[8]/46,sl,del(5)(q31q35),add(12)(p13)[7]/46,sl,add(12)(p13),del(17)(q21)[3]/46,XY,del(9)(q22q32)[2]	IDH1 R132; CKIT D816V; WT for IDH2, FLT3 and NPM1
2 Relapse/Refractory		76/F	45,XX,add(3)(q27),-7,add(8)(q24),t(9;22)(q34;q11.2)[4]/54,sl,+3,-add(3)(q27),+7,+8,+10,+12,+13,+15,+20,+der(22)t(9;22)(q34;q11.2)[16]	WT for FLT3, NPM1, CEBPA, IDH1 and IDH2
3 NA	NA	NA	NA	NA
4 NA	NA	NA	NA	NA
5 Relapse/Refractory	NA	NA	NA	FLT3 ITD, NPM1
6 NA	NA	NA	NA	NA
7 De novo AML	49/F		Normal karyotype (46, XX)	FLT3 ITD+; WT for CEBPA, NPM1, IDH1, IDH2, JAK2
8 Relapse/Refractory	49/F		46,XX[21]	FLT3 ITD+
9 De novo AML	52/M		45,XY,-7[3]/46,sl,+r(7)(p11q21)[11]/46,sl,der(5)t(1;5)(q31;p14)[5]/46,XY[1]	ASXL1, DNMT3a, NOTCH, NRAS
10 De novo AML	51/M		46,XY,add(1)(p11),del(5)(q15q33),del(7)(q22q36),der(11)t(1;11)(p31;p12-14)[20], Loss of 5q31 and 7q31	FLT3 ITD, BCOR, NOTCH1
11 De novo AML	70/F		46,XX[20] Normal	FLT3 TKD, NPM1
12 De novo AML	60/F		46,XX,t(9;11)(p21;q23)[13]/47,sl,+21[4]/47,sl,+8[3]	KRAS, PTPN11
13 De novo AML	66/F		46,XX,1~26dmin[11]/46,sl,add(3)(q2?6),add(8)(q24),4~70dmin[4]/46,sl,der(5)(q31),del(14)(q32)[2]/46,sl,der(14)(q32),+14,add(15)(q11.2)[2]/92,slx2[2]	TET2, ASXL1
14 De novo AML	56/F		46,XX[22] Normal	FLT3 ITD, DNMT3A, WT1, GATA2
15 De novo AML	63/F		46,XX[20] Normal	RUNX1, SF3B1, U2AF1
16 Relapse/Refractory	79/M		46,XY,t(6;9)(p21;q34)	FLT3 ITD, FLT3 TKD, IDH2
17 Relapse/Refractory	69/F		46,XX,add(14)(q22)[4]	FLT3, IDH1, NPM1

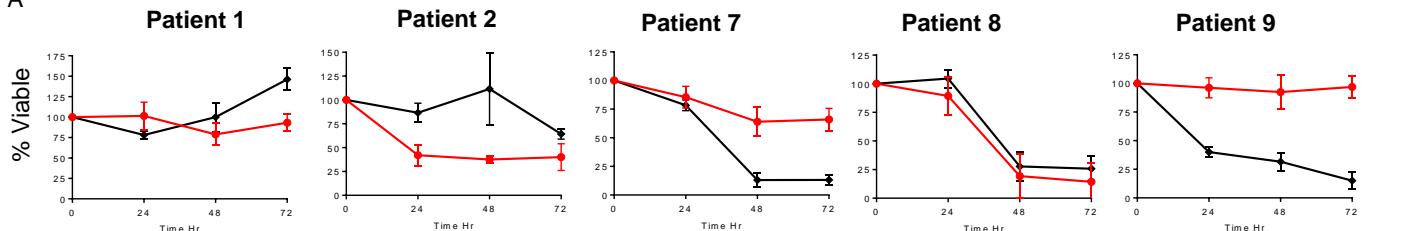
M=male, F=female, NA= not available

**Table S2: Significantly different metabolites in ROS-low LSCs vs. ROS-high AML blasts. Related to Figure 1.**

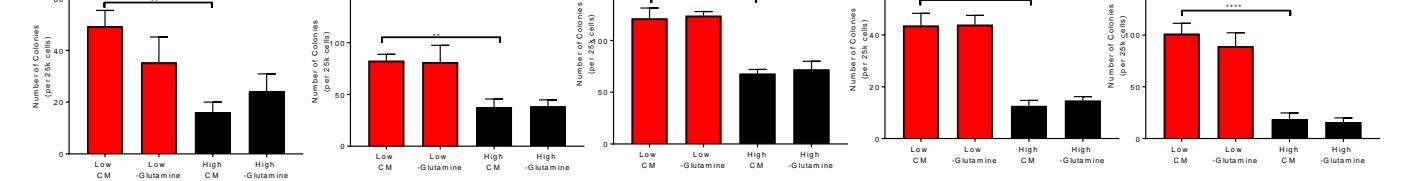
Metabolites Significantly More Abundant in ROS-low LSCs	t.stat	p.value	-LOG10(p)	FDR
alanine	6.31	0.0000	4.7135	0.0010
Ornithine	5.59	0.0001	4.1801	0.0015
histidine	5.45	0.0001	4.0646	0.0015
lysine	5.29	0.0001	3.9407	0.0015
serine	5.13	0.0002	3.8159	0.0017
leucine/isoleucine	4.96	0.0002	3.6813	0.0018
glutamine	4.90	0.0002	3.6304	0.0018
tryptophan	4.76	0.0003	3.5156	0.0021
5-Oxoproline	4.63	0.0004	3.4067	0.0024
threonine	4.41	0.0006	3.2294	0.0030
phenylalanine	4.34	0.0007	3.1650	0.0030
tyrosine	4.30	0.0007	3.1338	0.0030
arginine	4.24	0.0008	3.0867	0.0030
aspartate	4.24	0.0008	3.0800	0.0030
Xanthine	4.23	0.0008	3.0755	0.0030
valine	4.15	0.0010	3.0103	0.0033
Ethanolamine phosphate	4.07	0.0011	2.9406	0.0035
Nicotinamide	4.06	0.0012	2.9307	0.0035
proline	4.01	0.0013	2.8907	0.0037
methionine	3.74	0.0022	2.6562	0.0060
N-Acetylornithine	3.68	0.0025	2.6029	0.0064
L-Citrulline	3.63	0.0027	2.5651	0.0066
Adenine	3.62	0.0028	2.5523	0.0066
5-6-Dihydrothymine	3.59	0.0030	2.5294	0.0067
Creatine	3.46	0.0039	2.4140	0.0083
L-Methionine S-oxide	3.31	0.0052	2.2834	0.0102
glutamate	3.30	0.0053	2.2790	0.0102
Hypoxanthine	3.30	0.0053	2.2768	0.0102
cis-p-Coumarate	3.27	0.0056	2.2503	0.0105
Taurine	3.23	0.0060	2.2216	0.0108
Glutathione	3.15	0.0071	2.1466	0.0124
Malate	3.03	0.0091	2.0431	0.0153
L-Carnitine	2.75	0.0155	1.8097	0.0252
Riboflavin	2.74	0.0161	1.7933	0.0252
Diphosphate	2.73	0.0164	1.7861	0.0252
6-Lactoyl-5-6-7-8-tetrahydropterin	2.61	0.0205	1.6892	0.0307
O-Propanoylcarnitine	2.56	0.0227	1.6434	0.0332
Ascorbate	2.54	0.0238	1.6235	0.0338
Citrate	2.46	0.0276	1.5598	0.0382

● Low -Glutamine  
◆ High -Glutamine

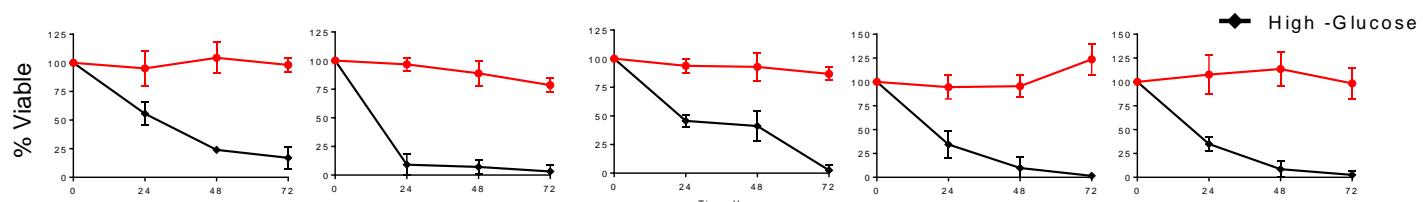
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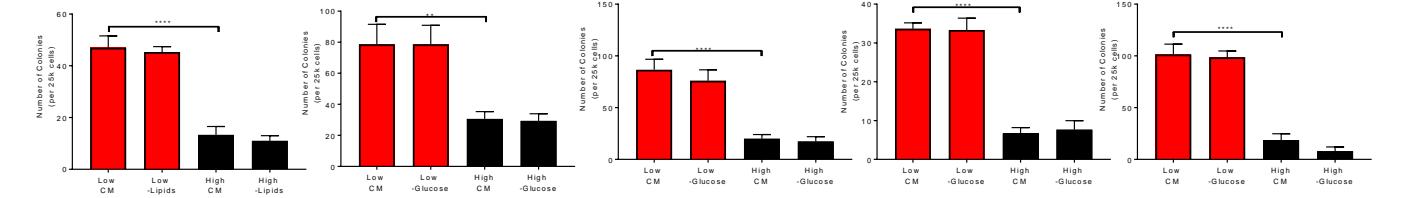
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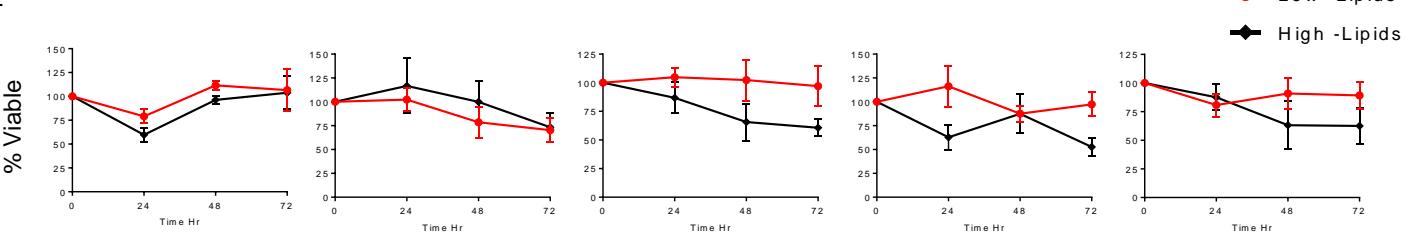
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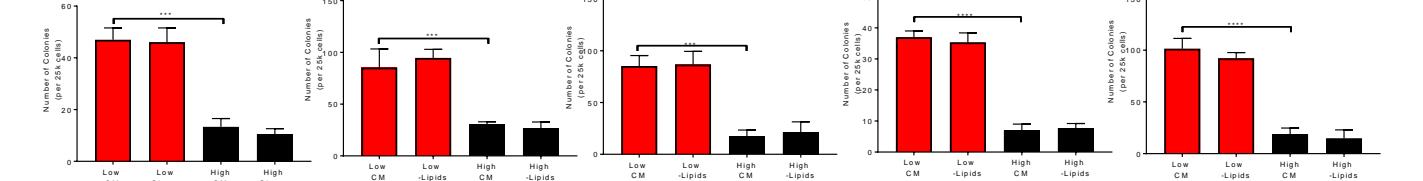
D



E



F



**Figure S2: ROS-low LSCs are not dependent on glutamine, glucose, or lipids. Related to Figure 2.**

**A.** Viability of ROS-low LSCs and ROS-high blasts after culturing without glutamine for 24, 48, and 72 hr.

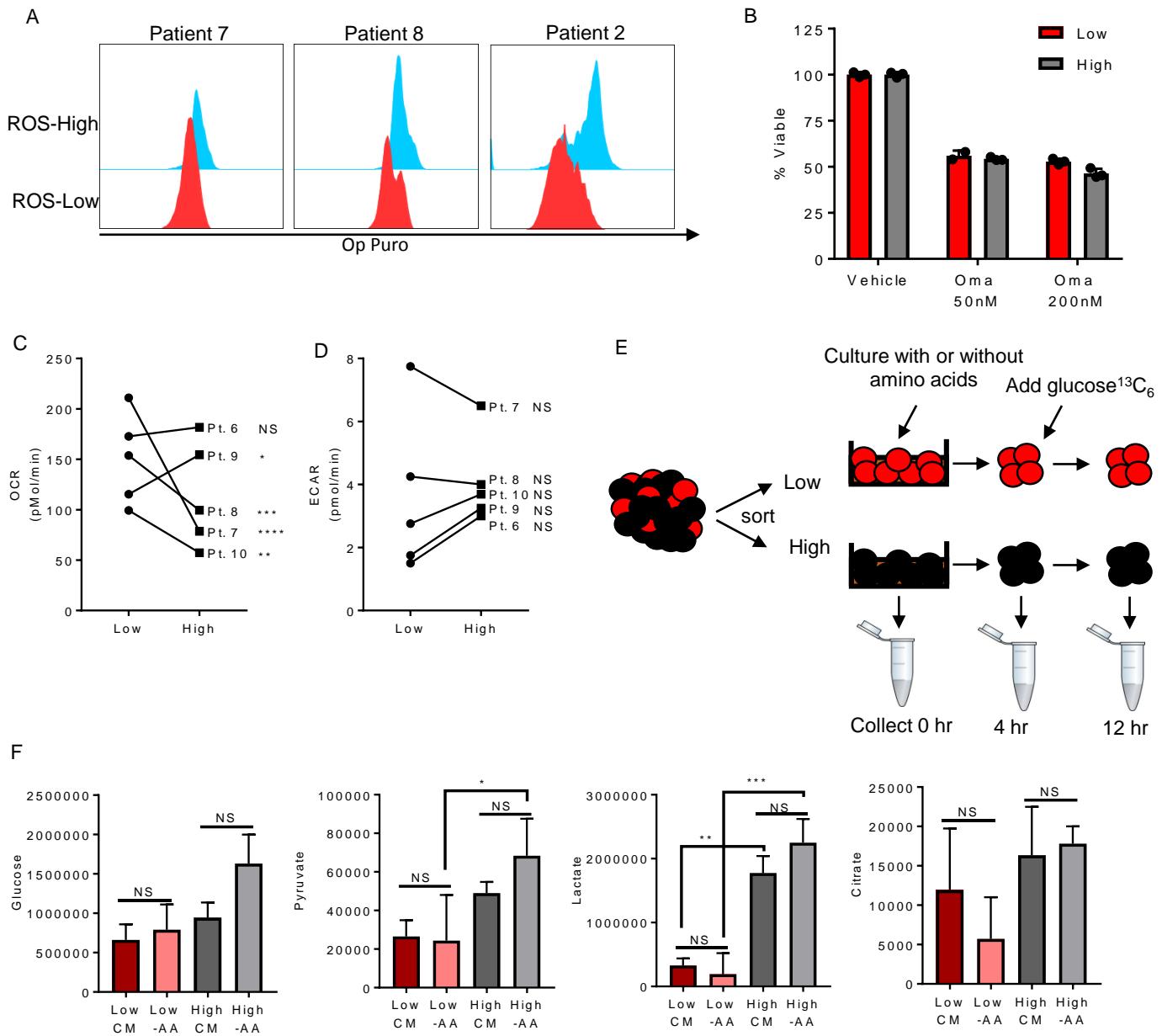
**B.** Colony-forming ability of ROS-low LSCs and ROS-high blasts after culturing without glutamine for 24 hr.

**C.** Viability of ROS-low LSCs and ROS-high blasts after culturing without glucose for 24, 48, and 72 hr.

**D.** Colony-forming ability of ROS-low LSCs and ROS-high blasts after culturing without glucose for 24 hr.

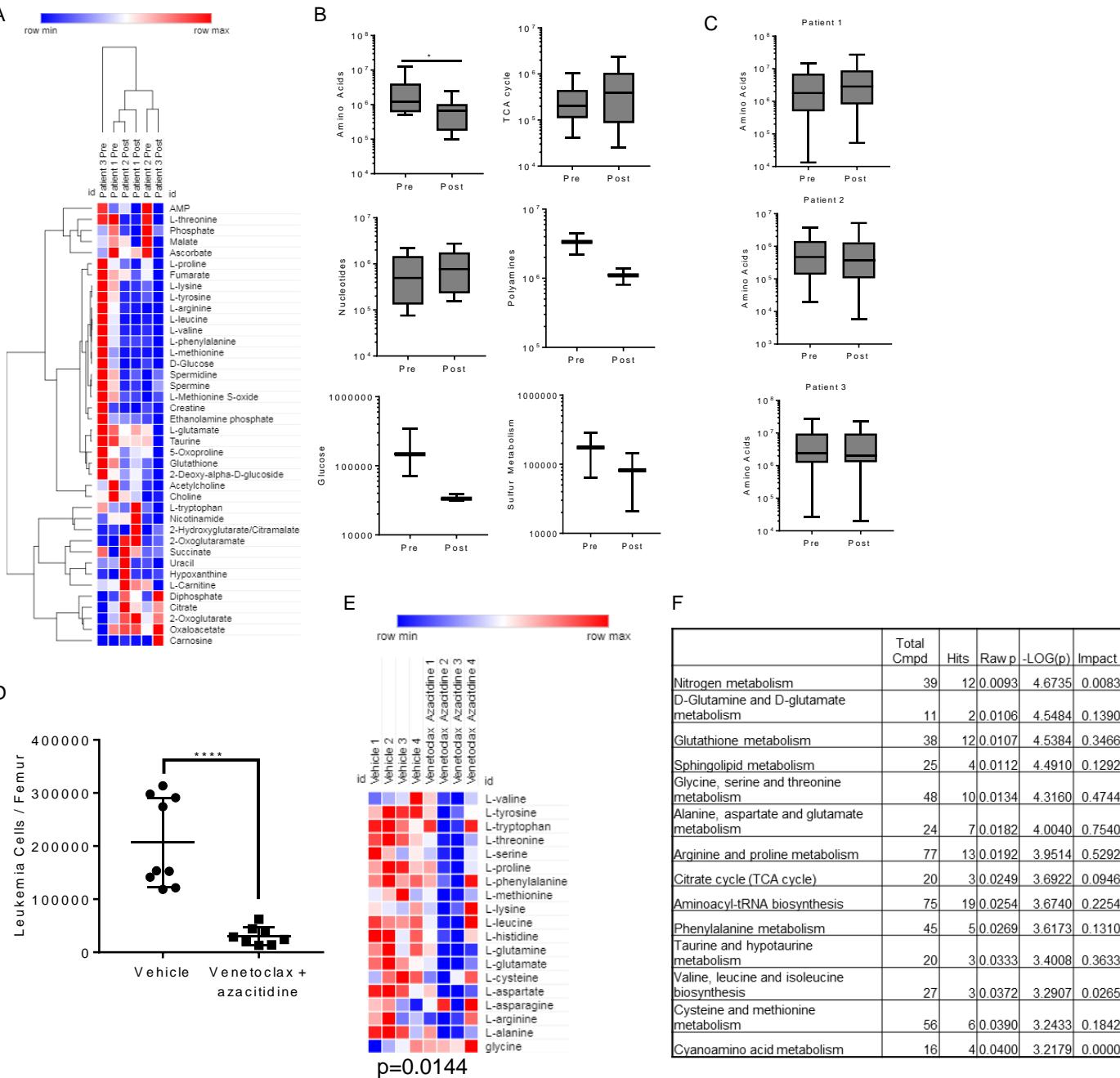
**E.** Viability of ROS-low LSCs and ROS-high blasts after culturing without lipids for 24, 48, and 72 hr.

**F.** Colony-forming ability of ROS-low LSCs and ROS-high blasts after culturing without lipids for 24 hr. **Each graph is relative to ROS-low LSCs cultured in complete media (CM), media containing all metabolites.** Each assay was repeated in triplicate. Graphs represent mean and error bars represent SD. Statistical analysis was performed using two-way Anova. \*\* p<0.01, \*\*\* p<0.005, \*\*\*\* p<0.001



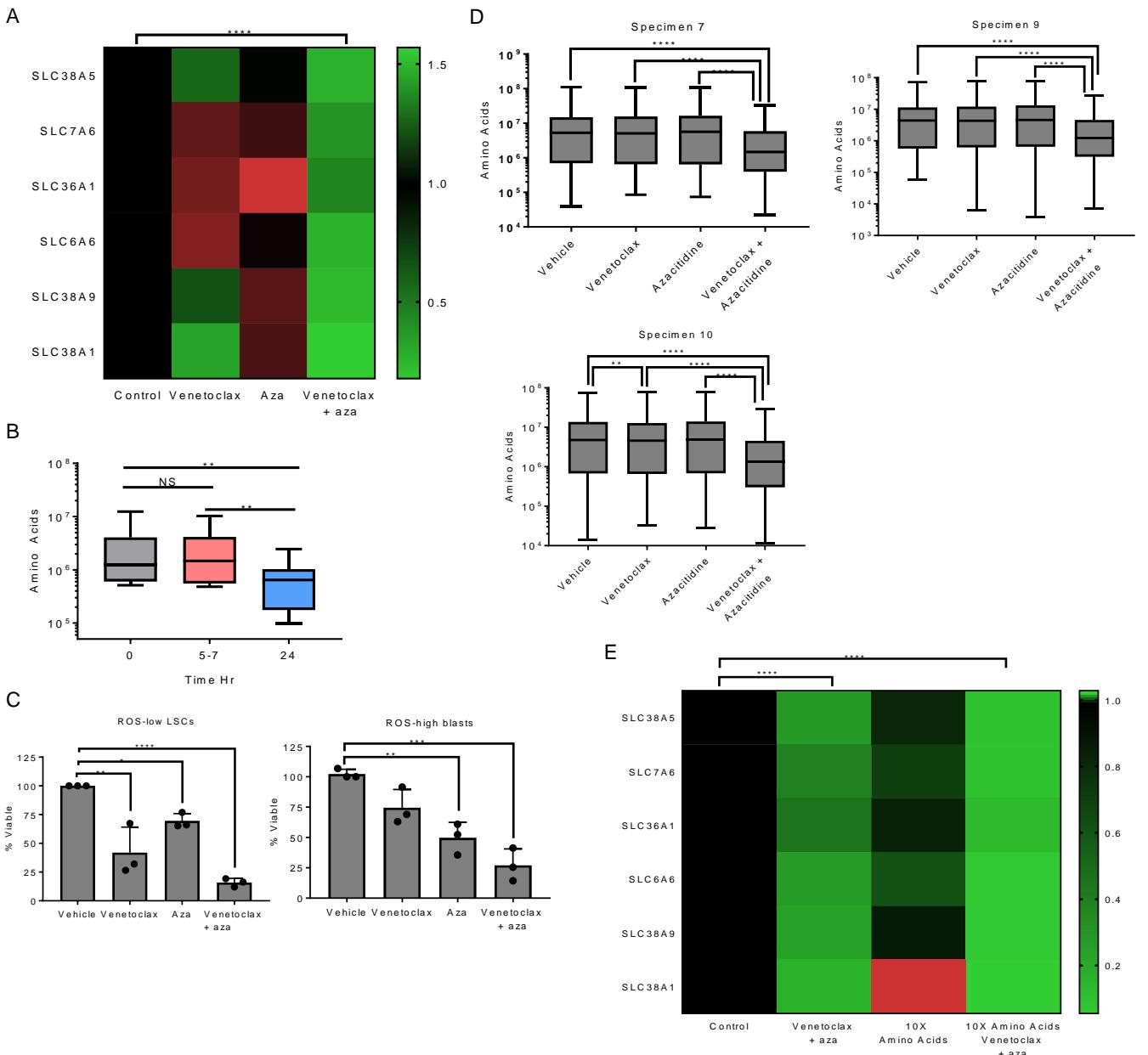
**Figure S3: Protein translation inhibition does not preferentially target LSCs. Related to Figure 3.**

**A.** Protein translation levels measured by OP-puro staining in ROS-low LSCs (red) and ROS-high cells (blue). **B.** Viability of ROS-Low LSCs and ROS-high cells isolated from patients 2 treated with Oma for 24 hr. **Relative to vehicle control treated cells.** **C.** Baseline levels of oxygen consumption OCR of ROS-low LSCs and ROS-high blasts. **Statistical significance was determined by unpaired two-tailed Student's t-test. (n=5)** **D.** Baseline levels of glycolysis (ECAR) of ROS-low LSCs and ROS-high blasts. **Statistical significance was determined by unpaired two-tailed Student's t-test. (n=5)** **E.** Diagram showing experimental design. ROS-low LSCs and ROS-high AML cells were cultured with or without amino acids for 4 hr. Stable isotope glucose was fluxed into cells for 8 hr. **F.** Graph shows levels of <sup>13</sup>C glucose and enrichment of heavy atom (<sup>13</sup>C) from the stable isotope glucose into TCA cycle intermediates. Specimen 7 used for this analysis. Statistical analysis was performed using two-way ANOVA. (n=4) \* p<0.05, \* p<0.01, \*\*\* p<0.005, \*\*\*\* p<0.001, NS = not significant



**Figure S4: Global Metabolic Changes in patients treated with venetoclax + azacitidine. Related to Figure 4.**

**A.** Heatmap showing the individual metabolites from three patient pre and 24 hr post venetoclax + azacitidine treatment. **B.** Metabolic pathways altered after a 24 hr treatment with venetoclax + azacitidine in patients. **Box plots represent the minimum to maximum values with the center line representing the mean. Error bars represent standard deviation.** **C.** Amino acid levels in the ROS-high AML blasts isolated from the 3 patients pre and post venetoclax with azacitidine. **Box plots represent the minimum to maximum values with the center line representing the mean. Error bars represent standard deviation. (n=4)** **D.** Leukemia cell burden in a patient derived xenograft (PDX) model treated with venetoclax (100mg/kg) and azacitidine (3mg/kg) for 2 weeks. Patient specimen 7 was used for this analysis. **Each dot represents the leukemia cells / femur in an individual mouse.** **E.** Heatmap showing amino acids with examples showing amino acid levels in AML cells isolated from the PDX model 24 hr post treatment with venetoclax with azacitidine. Patient specimen 7 was used for this analysis. **F.** Metabolic pathways identified as significantly changed after a 24 hr *in vivo* treatment with one dose of venetoclax (100mg/kg) + azacitidine (3mg/kg). Patient specimen 7 was used for this analysis. Statistical analysis was performed using Student's T-test. \* p<0.05, \*\*\*\* p<0.001

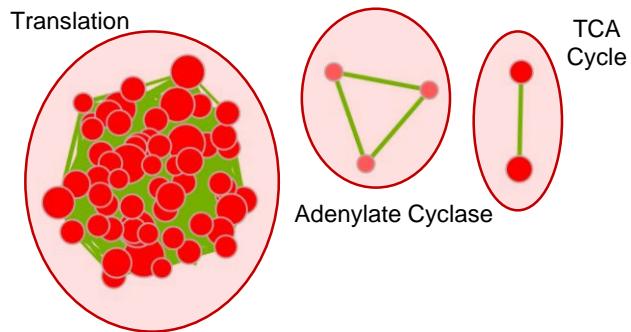


**Figure S5: Amino Acid changes upon Venetoclax with azacitidine treatment over time. Related to Figure 5.**

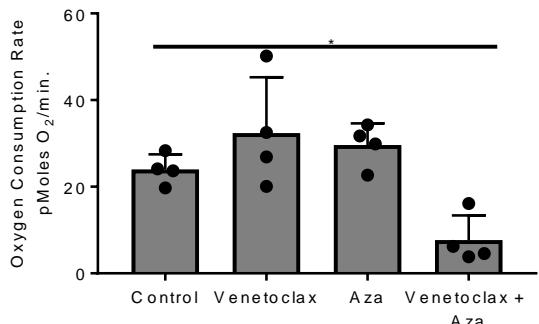
**A.** Expression of amino acid transporters in ROS-low LSCs isolated from specimens 7, 9, and 10 treated with 500 nM venetoclax, 2.5  $\mu$ M azacitidine, or the combination of the two for 4 hr. **B.** Amino acid levels from the 3 patients treated with venetoclax + azacitidine for 0 hr, 5 or 7 hr, and 24 hr. Graph represents the combined changes of the three patients. **Box plots represent the minimum to maximum values with the center line representing the mean. Error bars represent standard deviation.** **C.** Viability of ROS-low LSCs and ROS-high AML blasts isolated from specimens 7, 9, and 10 treated with 500 nM venetoclax, 2.5  $\mu$ M azacitidine, or the combination of the two for 24 hr. **Viability is relative to vehicle control.** **D.** Amino acid levels measured in ROS-low LSCs isolated from specimens 7, 9, and 10 treated with 500 nM venetoclax, 2.5  $\mu$ M azacitidine, or the combination of the two for 4 hr. **Box plots represent the minimum to maximum values with the center line representing the mean. Error bars represent standard deviation.** **E.** Expression of amino acid transporters in ROS-low LSCs isolated from specimens 7, 9, and 10 pretreated with 10 times normal levels of amino acids for 2 hr and then treated with 500 nM venetoclax and 2.5  $\mu$ M azacitidine for 4 hr. **Graphs represent mean and error bars represent SD.** Statistical analysis was performed using two-way Anova analysis. \* p<0.05, \*\* p<0.01, \*\*\* p<0.005, \*\*\*\* p<0.001, NS = not significant

A

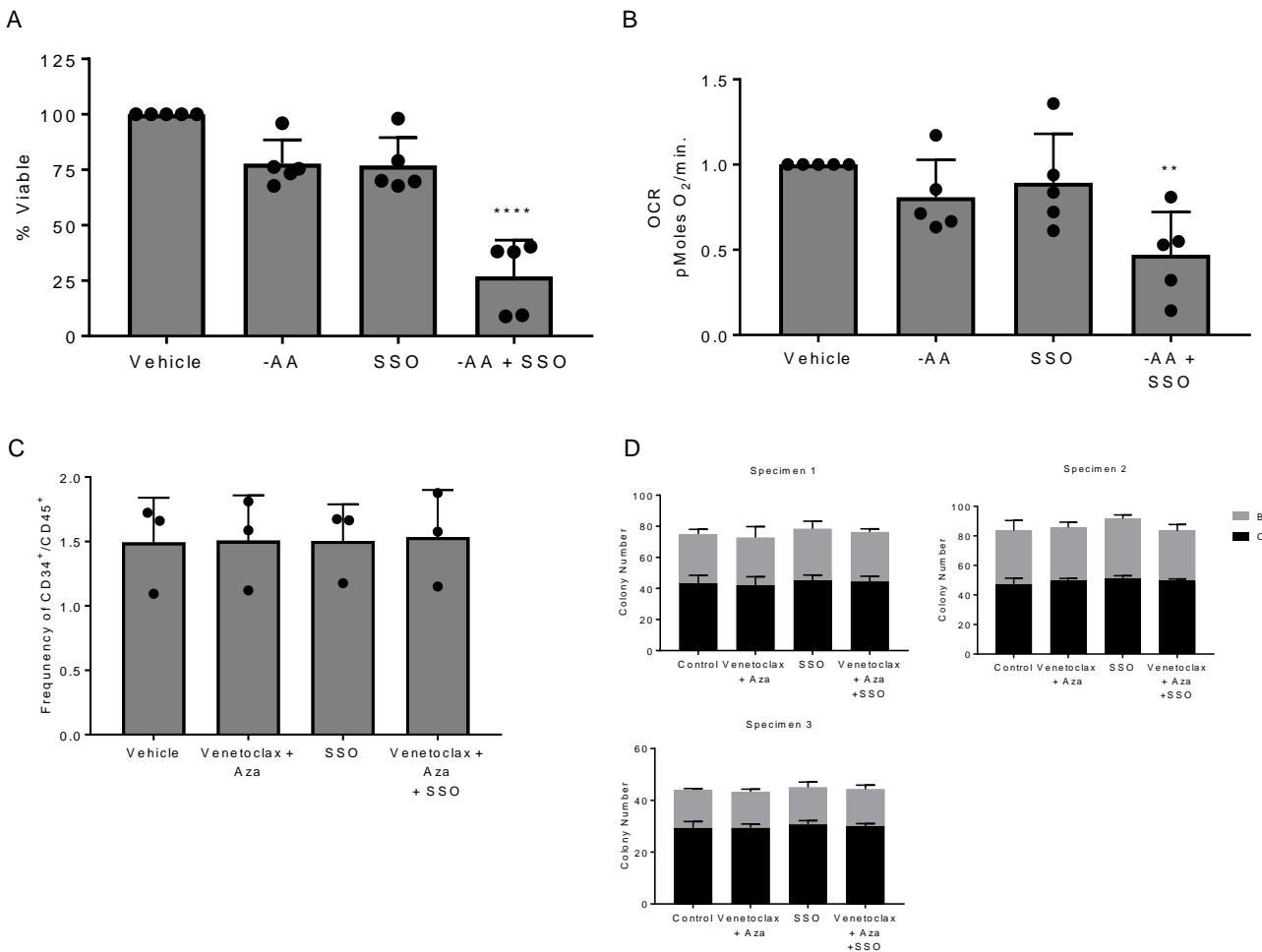
## Downregulated pathways in venetoclax + azacitidine



B

**Figure S6: Venetoclax with azacitidine decreases OXPHOS *in vivo*. Related to Figure 6.**

**A.** Enrichment map analysis of RNA-sequencing from ROS-low LSCs isolated from 3 patients treated with venetoclax with azacitidine on the clinical trial. **B.** OXPHOS levels in blasts isolated from a patient derived xenograft (PDX) model treated with venetoclax (100mg/kg) and azacitidine (3mg/kg) for 24 hr. Specimen 7 was used in this analysis. Statistical analysis was performed using two-way Anova. \* p<0.05



**Figure S7: Fatty acid metabolism inhibition targets LSCs but not HSCs in combination with amino acid inhibition. Related to Figure 7.**

**A.** Viability of LSCs isolated from relapse/refractory AML patients and cultured without amino acids, 50  $\mu$ M SSO, or without amino acids and SSO for 24 hr **relative to vehicle treatment**. **B.** OXPHOS levels in LSCs isolated from relapse/refractory AML patients and cultured without amino acids, 50  $\mu$ M SSO, or without amino acids and SSO for 4 hr. Each dot represents an individual patient sample treated *in vitro*. Specimens 1, 2, 8, 16 and 17 were used for analysis in A and B. **C.** Percentage of normal CD34+/CD45+ cells in mobilized peripheral blood after treatment with 500 nM venetoclax with 2.5  $\mu$ M azacitidine, 50  $\mu$ M SSO, or venetoclax with azacitidine and SSO for 24 hr. **Each dot represents a individual patient specimen.** **D.** Colony forming ability of mobilized peripheral blood after treatment with 500 nM venetoclax with 2.5  $\mu$ M azacitidine, 50  $\mu$ M SSO, or venetoclax with azacitidine and SSO for 24 hr. **CFU-GM = colony forming unit - granulocyte/monocyte, BFU-E = burst forming unit - erythroid.** (n=3) Statistical analysis was performed using two-way Anova. \*\* p<0.01, \*\*\*\* p<0.0001